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(71) Applicant (for all designated States except US): **DE-GUSSA AG [DE/DE]**; Bennigsenplatz 1, 40474 Düsseldorf (DE).

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(72) Inventor; and

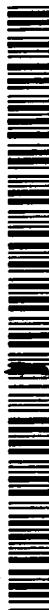
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**WO 03/008607 A2**

(54) Title: PROCESS FOR THE PREPARATION OF L-AMINO ACIDS USING STRAINS OF THE ENTEROBACTERIACEAE FAMILY

(57) Abstract: The invention relates to a process for the preparation of L-amino acids, in particular L-threonine, in which the following steps are carried out: a) fermentation of microorganisms of the Enterobacteriaceae family which produce the desired L-amino acid and in which at least one or more of the genes chosen from the group consisting of phoA, phnC, phnD, phnE, phnF, phnG, phnJ, phnK, phnL, phnM, phnN, phnO, phnP, eda, rpiB, zwf, mopA, pstA, pstB, pstC, pstS, ugpB, ugpA, ugpE, ugpC, ugpQ, dnaK, dnaJ, clpB, rpoE, htpG, ompF, ompC, aspA, gltA, sdhB, aceB and aceK, or nucleotide sequences which code for these, is or are enhanced, in particular over-expressed, b) concentration of the desired L-amino acid in the medium or in the cells of the bacteria, and c) isolation of the desired L-amino acid.

**Process for the Preparation of L-Amino Acids using Strains  
of the Enterobacteriaceae Family**

**Field of the Invention**

This invention relates to a process for the preparation of  
5 L-amino acids, in particular L-threonine, using strains of  
the Enterobacteriaceae family in which at least one or more  
of the genes chosen from the group consisting of phoA,  
phnC, phnD, phnE, phnF, phnG, phnJ, phnK, phnL, phnM, phnN,  
phnO, phnP, eda, rpiB, zwf, mopA, pstA, pstB, pstC, pstS,  
10 ugpB, ugpA, ugpE, ugpC, ugpQ, dnaK, dnaJ, clpB, rpoE, htpG,  
ompF, ompC, aspA, gltA, sdhB, aceB and aceK is (are)  
enhanced.

**Prior Art**

L-Amino acids, in particular L-threonine, are used in human  
15 medicine and in the pharmaceuticals industry, in the  
foodstuffs industry and very particularly in animal  
nutrition.

It is known to prepare L-amino acids by fermentation of  
strains of Enterobacteriaceae, in particular Escherichia  
20 coli (E. coli) and Serratia marcescens. Because of their  
great importance, work is constantly being undertaken to  
improve the preparation processes. Improvements to the  
process can relate to fermentation measures, such as e.g.  
stirring and supply of oxygen, or the composition of the  
25 nutrient media, such as e.g. the sugar concentration during  
the fermentation, or the working up to the product form, by  
e.g. ion exchange chromatography, or the intrinsic output  
properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are  
30 used to improve the output properties of these  
microorganisms. Strains which are resistant to  
antimetabolites, such as e.g. the threonine analogue α-  
amino-β-hydroxyvaleric acid (AHV), or are auxotrophic for

metabolites of regulatory importance and produce L-amino acid, such as e.g. L-threonine, are obtained in this manner.

Methods of the recombinant DNA technique have also been  
5 employed for some years for improving the strain of strains of the Enterobacteriaceae family which produce L-amino acids, by amplifying individual amino acid biosynthesis genes and investigating the effect on the production.

#### 10 Object of the Invention

The object of the invention is to provide new measures for improved fermentative preparation of L-amino acids, in particular L-threonine.

#### Summary of the Invention

15 The invention provides a process for the fermentative preparation of L-amino acids, in particular L-threonine, using microorganisms of the Enterobacteriaceae family which in particular already produce L-amino acids and in which at least one or more of the nucleotide sequence(s) which  
20 code(s) for the genes phoA, phnC, phnD, phnE, phnF, phnG, phnJ, phnK, phnL, phnM, phnN, phnO, phnP, eda, rpiB, zwf, mopa, pstA, pstB, pstC, pstS, ugpB, ugpA, ugpE, ugpC, ugpQ, dnaK, dnaJ, clpB, rpoE, htpG, ompF, ompC, aspA, gltA, sdhB, aceB and aceK is (are) enhanced.

#### 25 Detailed Description of the Invention

Where L-amino acids or amino acids are mentioned in the following, this means one or more amino acids, including their salts, chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine,  
30 L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-

histidine, L-lysine, L-tryptophan and L-arginine. L-Threonine is particularly preferred.

The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or a gene or allele which codes for a corresponding enzyme or protein with a high activity, and optionally combining 10 these measures.

By enhancement measures, in particular over-expression, the activity or concentration of the corresponding protein is in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or 15 2000%, based on that of the wild-type protein or the activity or concentration of the protein in the starting microorganism.

The process comprises carrying out the following steps:

- a) fermentation of microorganisms of the Enterobacteriaceae family in which one or more of the genes chosen from the group consisting of phoA, phnC, phnD, phnE, phnF, phnG, phnJ, phnK, phnL, phnM, phnN, phnO, phnP, eda, rpiB, zwf, mopA, pstA, pstB, pstC, pstS, ugpB, ugpA, ugpE, ugpC, ugpQ, dnaK, dnaJ, clpB, rpoE, htpG, ompF, ompC, aspA, gltA, sdhB, aceB and aceK is (are) enhanced,
- b) concentration of the corresponding L-amino acid in the medium or in the cells of the microorganisms of the Enterobacteriaceae family, and
- c) isolation of the desired L-amino acid, constituents of the fermentation broth and/or the biomass in its entirety or portions (> 0 to 100 %) thereof optionally remaining in the product.

The microorganisms which the present invention provides can produce L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, optionally starch, optionally cellulose or from glycerol and ethanol. They are

- 5 representatives of the Enterobacteriaceae family chosen from the genera Escherichia, Erwinia, Providencia and Serratia. The genera Escherichia and Serratia are preferred. Of the genus Escherichia the species Escherichia coli and of the genus Serratia the species Serratia
- 10 marcescens are to be mentioned in particular.

Suitable strains, which produce L-threonine in particular, of the genus Escherichia, in particular of the species Escherichia coli, are, for example

- 15 Escherichia coli TF427  
Escherichia coli H4578  
Escherichia coli KY10935  
Escherichia coli VNIIgenetika MG442  
Escherichia coli VNIIgenetika M1  
Escherichia coli VNIIgenetika 472T23
- 20 Escherichia coli BKIIM B-3996  
Escherichia coli kat 13  
Escherichia coli KCCM-10132.

Suitable L-threonine-producing strains of the genus Serratia, in particular of the species Serratia marcescens,  
25 are, for example

- Serratia marcescens HNr21  
Serratia marcescens TLr156  
Serratia marcescens T2000.

Strains from the Enterobacteriaceae family which produce L-  
30 threonine preferably have, inter alia, one or more genetic or phenotypic features chosen from the group consisting of: resistance to  $\alpha$ -amino- $\beta$ -hydroxyvaleric acid, resistance to thialysine, resistance to ethionine, resistance to Cr-

- methylserine, resistance to diaminosuccinic acid, resistance to  $\alpha$ -aminobutyric acid, resistance to borrelidin, resistance to rifampicin, resistance to valine analogues, such as, for example, valine hydroxamate,
- 5 resistance to purine analogues, such as, for example, 6-dimethylaminopurine, a need for L-methionine, optionally a partial and compensatable need for L-isoleucine, a need for meso-diaminopimelic acid, auxotrophy in respect of threonine-containing dipeptides, resistance to L-threonine,
- 10 resistance to L-homoserine, resistance to L-lysine, resistance to L-methionine, resistance to L-glutamic acid, resistance to L-aspartate, resistance to L-leucine, resistance to L-phenylalanine, resistance to L-serine, resistance to L-cysteine, resistance to L-valine,
- 15 sensitivity to fluoropyruvate, defective threonine dehydrogenase, optionally an ability for sucrose utilization, enhancement of the threonine operon, enhancement of homoserine dehydrogenase I-aspartate kinase I, preferably of the feed back resistant form, enhancement
- 20 of homoserine kinase, enhancement of threonine synthase, enhancement of aspartate kinase, optionally of the feed back resistant form, enhancement of aspartate semialdehyde dehydrogenase, enhancement of phosphoenol pyruvate carboxylase, optionally of the feed back resistant form,
- 25 enhancement of phosphoenol pyruvate synthase, enhancement of transhydrogenase, enhancement of the RhtB gene product, enhancement of the RhtC gene product, enhancement of the YfiK gene product, enhancement of a pyruvate carboxylase, and attenuation of acetic acid formation.
- 30 It has been found that microorganisms of the Enterobacteriaceae family produce L-amino acids, in particular L-threonine, in an improved manner after enhancement, in particular over-expression, of at least one or more of the genes chosen from the group consisting of
- 35 phoA, phnC, phnD, phnE, phnF, phnG, phnJ, phnK, phnL, phnM, phnN, phnO, phnP, eda, rpiB, zwf, mopA, pstA, pstB, pstC,

pstS, ugpB, ugpA, ugpE, ugpC, ugpQ, dnaK, dnaJ, clpB, rpoE, htpG, ompF, ompC, aspA, gltA, sdhB, aceB and aceK.

The nucleotide sequences of the genes of *Escherichia coli* belong to the prior art and can also be found in the genome  
5 sequence of *Escherichia coli* published by Blattner et al.  
(*Science* 277: 1453-1462 (1997)).

phoA gene:

Description: Alkaline phosphatase

EC No.: 3.1.3.1

10 Reference: Berg; *Journal of Bacteriology* 146(2): 660-667 (1981)

Accession No.: AE000145

phnC gene:

Description: ATP-binding protein of the alkyl  
15 phosphonate transport system

Reference: Makino et al.; *Journal of Bacteriology*  
173(8): 2665-2672 (1991)

Accession No.: AE000482

phnD gene:

20 Description: Substrate-binding protein of the alkyl  
phosphonate transport system

Reference: Makino et al.; *Journal of Bacteriology*  
173(8): 2665-2672 (1991)

Accession No.: AE000482

25 Alternative gene name: psID

phnE gene:

Description: Permease protein of the alkyl phosphonate  
transport system

Reference: Makino et al.; *Journal of Bacteriology*  
30 173(8): 2665-2672 (1991)

Accession No.: AE000482

phnF gene:

Description: Putative regulatory protein

Reference: Makino et al.; Journal of Bacteriology  
173(8): 2665-2672 (1991)

Accession No.: AE000482

phnG gene:

5 Description: Membrane-bound sub-unit of the carbon-phosphorus lyase complex

Reference: Makino et al.; Journal of Bacteriology  
173(8): 2665-2672 (1991)

Accession No.: AE000482

10 phnJ gene:

Description: Membrane-bound sub-unit of the carbon-phosphorus lyase complex

Reference: Makino et al.; Journal of Bacteriology  
173(8): 2665-2672 (1991)

15 Accession No.: AE000482

phnK gene:

Description: ATP-binding protein of the phosphonate transporter

Reference: Makino et al.; Journal of Bacteriology  
20 173(8): 2665-2672 (1991)

Accession No.: AE000482

phnL gene:

Description: ATP-binding protein of the phosphonate transporter

25 Reference: Makino et al.; Journal of Bacteriology  
173(8): 2665-2672 (1991)

Accession No.: AE000482

phnM gene:

Description: Membrane-bound sub-unit of the carbon-phosphorus lyase complex

30 Reference: Makino et al.; Journal of Bacteriology  
173(8): 2665-2672 (1991)

Accession No.: AE000482

phmN gene:

Description: ATP-binding protein of the phosphonate transporter

Reference: Makino et al.; Journal of Bacteriology  
5 173(8): 2665-2672 (1991)

Accession No.: AE000482

phmO gene:

Description: Putative regulator of the carbon-phosphorus lyase complex

10 Reference: Makino et al.; Journal of Bacteriology  
173(8): 2665-2672 (1991)

Accession No.: AE000482

phmP gene:

Description: Membrane-bound sub-unit of the carbon-phosphorus lyase complex

15 Reference: Makino et al.; Journal of Bacteriology  
173(8): 2665-2672 (1991)

Accession No.: AE000482

eda gene:

20 Description: 2-Keto-3-deoxygluconate 6-phosphate aldolase and 2-keto-4-hydroxyglutarate aldolase (Entner-Doudoroff aldolase)

EC No.: 4.1.2.14

4.1.3.16

25 Reference: Carter et al.; Gene 130(1): 155-156 (1993)

Accession No.: AE000279

Alternative gene names: hga, kdgA

rpiB gene:

Description: Ribose 5-phosphate isomerase B

30 EC No.: 5.3.1.6

Reference: Sorensen und Hove-Jensen; Journal of Bacteriology 178(4): 1003-1011 (1996)

Accession No.: AE000482

zwf gene:

Description: Glucose 6-phosphate 1-dehydrogenase

EC No.: 1.1.1.49

Reference: Rowley and Wolf; Journal of Bacteriology

5 173(3): 968-977 (1991)

Accession No.: AE000279

mopA gene:

Description: Chaperone GroEL, heat shock protein Hsp60

Reference: Chandrasekhar et al.; Journal of Biological  
10 Chemistry 261(26): 12414-12419 (1986)

Accession No.: AE000487

Alternative gene names: groE, groEL, hdh, tabB

pstA gene:

Description: Permease protein of the high-affinity  
15 phosphate transport system

Reference: Surin et al.; Journal of Bacteriology  
161(1): 189-198 (1985); Amemura et al.;  
Journal of Molecular Biology 184(2): 241-  
250 (1985)

20 Accession No.: AE000449

Alternative gene names: R2pho, phoR2b, phoT

pstB gene:

Description: ATP-binding component of the high-affinity  
phosphate transport system

25 Reference: Surin et al.; Journal of Bacteriology  
161(1): 189-198 (1985); Amemura et al.;  
Journal of Molecular Biology 184(2): 241-  
250 (1985)

Accession No.: AE000449

30 Alternative gene name: phoT

pstC gene:

Description: Permease protein of the high-affinity  
phosphate transport system

Reference: Surin et al.; Journal of Bacteriology  
161(1): 189-198 (1985)

Accession No.: AE000449

Alternative gene name: phoW

5 pstS gene:

Description: Periplasmic phosphate-binding protein of  
the high-affinity phosphate transport  
system

Reference: Surin et al., Journal of Bacteriology  
10 157(3): 772-778 (1984); Magota et al.,  
Journal of Bacteriology 157(3): 909-917  
(1984)

Accession No.: AE000449

Alternative gene names: R2pho, nmpA, phoR2a, phoS

15 ugpB gene:

Description: Periplasmic binding protein of the sn-  
glycerol 3-phosphate transport system

Reference: Overduin et al.; Molecular Microbiology  
20 2(6): 767-775 (1988); Xavier et al.;  
Journal of Bacteriology 177(3): 699-704  
(1995); Schweizer and Boos; Molecular and  
General Genetics 197(1): 161-168 (1984)

Accession No.: AE000421

Alternative gene names: psiB, psiC,

25 ugpA gene:

Description: Permease protein of the sn-glycerol 3-  
phosphate transport system

Reference: Overduin et al.; Molecular Microbiology  
30 2(6): 767-775 (1988); Xavier et al.;  
Journal of Bacteriology 177(3): 699-704  
(1995)

Accession No.: AE000421

Alternative gene names: psiB, psiC

ugpE gene:

Description: Permease protein of the sn-glycerol 3-phosphate transport system

Reference: Overduin et al.; Molecular Microbiology 5 2(6): 767-775 (1988); Xavier et al.; Journal of Bacteriology 177(3): 699-704 (1995)

Accession No.: AE000421

ugpC gene:

10 Description: ATP-binding component of the sn-glycerol 3-phosphate transport system

Reference: Overduin et al.; Molecular Microbiology 15 2(6): 767-775 (1988); Xavier et al.; Journal of Bacteriology 177(3): 699-704 (1995); Hekstra and Tommassen; Journal of Bacteriology 175(20): 6546-6552 (1993)

Accession No.: AE000421

ugpQ gene:

Description: Glycerol phosphodiester phosphodiesterase

20 EC No.: 3.1.4.46

Reference: Kasahara et al.; Nucleic Acids Research 17(7): 2854 (1989); Brzoska and Boos; FEMS Microbiology Reviews 5(1-2): 115-124 (1989)

Accession No.: AE000421

25 dnaK gene:

Description: Autoregulated heat shock protein Hsp70

Reference: Bardwell and Craig; Proceedings of the National Academy of Sciences of the United States of America 81(3): 848-52 (1984)

30 Accession No.: AE000112

Alternative gene names: gro, groP, groPAB, groPC, groPF, rpC, grpF, seg

dnaJ gene:

Description: Chaperone; heat shock protein

Reference: Ohki et al.; Journal of Biological Chemistry 261(4): 1778-1781 (1986)

Accession No.: AE000112

Alternative gene names: groP, grpC

5 clpB gene:

Description: Heat shock protein f84.1

Reference: Kitagawa et al.; Journal of Bacteriology 173(14): 4247-4253 (1991)

Accession No.: AE000345

10 Alternative gene name: htpM

rpoE gene:

Description: Sigma-E sub-unit of RNA polymerase

Reference: Raina et al.; EMBO Journal 14(5): 1043-1055 (1995)

15 Accession No.: AE000343

Alternative gene name: sigE

htpG gene:

Description: Chaperone; heat shock protein c62.5

Reference: Spence and Georgopoulos; Journal of Biological Chemistry 264(8): 4398-4403 (1989); Bardwell and Craig; Proceedings of the National Academy of Sciences of the United States of America 84(15): 5177-5181 (1987)

25 Accession No.: AE000153

ompF gene:

Description: Outer membrane protein F (= 1a; ia; B)

Reference: Inokuchi et al.; Nucleic Acids Research 10(21): 6957-6968 (1982)

30 Accession No.: AE000195

Alternative gene names: cmlB, coa, cry, tolF

ompC gene:

Description: Outer membrane protein C (= 1b)

Reference: Mizuno et al.; Journal of Biological Chemistry 258(11): 6932-6940 (1983)

Accession No.: AE000310

Alternative gene names: meoA, par

5 aspA gene:

Description: Aspartate ammonium lyase (aspartase)

EC No.: 4.3.1.1

Reference: Takagi et al.; Nucleic Acids Research

10 13(6): 2063-2074 (1985); Woods et al.;  
Biochemical Journal 237(2): 547-557 (1986);  
Falzone et al.; Biochemistry 27(26): 9089-  
9093 (1988); Jayasekera et al.;  
Biochemistry 36(30): 9145-9150 (1997).

Accession No.: AE000486

15 gltA gene:

Description: Citrate synthase

EC No.: 4.1.3.7

Reference: Spencer and Guest, Journal of Bacteriology  
151(2): 542-552 (1982)

20 Accession No.: AE000175

Alternative gene names: gluT, icdB

sdhB gene:

Description: Iron-sulfur protein sub-unit of succinate  
dehydrogenase

25 EC No.: 1.3.99.1

Reference: Darlison and Guest, Biochemical Journal  
223(2), 507-517 (1984)

Accession No.: AE000175

aceB gene:

30 Description: Malate synthase A

EC No.: 4.1.3.2

Reference: Byrne et al.; Nucleic Acids Research 16(19), 9342 (1988); Byrne et al.; Nucleic Acids Research 16(22), 10924 (1988); Cortay et al.; Biochimie 71(9-10): 1043-9 (1989)

5 Accession No.: AE000474

Alternative gene name: mas

aceK gene:

Description: Isocitrate dehydrogenase kinase/phosphatase

EC No.: 2.7.1.116

10 3.1.3.-

Reference: Cortay et al.; Journal of Bacteriology 170(1): 89-97 (1988); Klumpp et al.; Journal of Bacteriology 170(6): 2763-2769 (1988); Nelson et al.; Genetics 147(4):

15 1509-1520 (1997); Laporte et al.; Biochimie 71(9-10): 1051-7 (1989)

Accession No.: AE000474

The nucleic acid sequences can be found in the databanks of the National Center for Biotechnology Information (NCBI) of 20 the National Library of Medicine (Bethesda, MD, USA), the nucleotide sequence databank of the European Molecular Biologies Laboratories (EMBL, Heidelberg, Germany or Cambridge, UK) or the DNA databank of Japan (DDBJ, Mishima, Japan).

25 The genes described in the text references mentioned can be used according to the invention. Alleles of the genes which result from the degeneracy of the genetic code or due to "sense mutations" of neutral function can furthermore be used.

30 To achieve an enhancement, for example, expression of the genes or the catalytic properties of the proteins can be increased. The two measures can optionally be combined.

- To achieve an over-expression, the number of copies of the corresponding genes can be increased, or the promoter and regulation region or the ribosome binding site upstream of the structural gene can be mutated. Expression cassettes which are incorporated upstream of the structural gene act in the same way. By inducible promoters, it is additionally possible to increase the expression in the course of fermentative L-threonine production. The expression is likewise improved by measures to prolong the life of the m-RNA. Furthermore, the enzyme activity is also increased by preventing the degradation of the enzyme protein. The genes or gene constructs can either be present in plasmids with a varying number of copies, or can be integrated and amplified in the chromosome.
- Alternatively, an over-expression of the genes in question can furthermore be achieved by changing the composition of the media and the culture procedure.

Instructions in this context can be found by the expert, inter alia, in Chang and Cohen (Journal of Bacteriology 20 134: 1141-1156 (1978)), in Hartley and Gregori (Gene 13: 347-353 (1981)), in Amann and Brosius (Gene 40: 183-190 (1985)), in de Broer et al. (Proceedings of the National Academy of Sciences of the United States of America 80: 21-25 (1983)), in LaVallie et al. (BIO/TECHNOLOGY 11: 187-193 (1993)), in PCT/US97/13359, in Llosa et al. (Plasmid 26: 222-224 (1991)), in Quandt and Klipp (Gene 80: 161-169 (1989)), in Hamilton et al. (Journal of Bacteriology 171: 4617-4622 (1989)), in Jensen and Hammer (Biotechnology and Bioengineering 58: 191-195 (1998)) and in known textbooks of genetics and molecular biology.

Plasmid vectors which are capable of replication in Enterobacteriaceae, such as e.g. cloning vectors derived from pACYC184 (Bartolomé et al.; Gene 102: 75-78 (1991)), pTrc99A (Amann et al.; (Gene 69: 301-315 (1988)) or pSC101 derivatives (Vocke and Bastia, Proceedings of the National

Academy of Sciences USA 80 (21): 6557-6561 (1983)) can be used. A strain transformed with a plasmid vector, where the plasmid vector carries at least one or more of the genes chosen from the group consisting of phoA, phnC, phnD,  
5 phnE, phnF, phnG, phnJ, phnK, phnL, phnM, phnN, phnO, phnP, eda, rpiB, zwf, mopA, pstA, pstB, pstC, pstS, ugpB, ugpA, ugpE, ugpC, ugpQ, dnaK, dnaJ, clpB, rpoE, htpG, ompF, ompC, aspA, gltA, sdhB, aceB and aceK, or nucleotide sequences which code for these, can be employed in a process  
10 according to the invention.

It is also possible to transfer mutations which affect the expression of the particular gene into various strains by sequence exchange (Hamilton et al.; Journal of Bacteriology 171: 4617-4622 (1989)), conjugation or transduction.

15 It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, with strains of the Enterobacteriaceae family, in addition to enhancement of one or more of the genes chosen from the group consisting of phoA, phnC, phnD, phnE, phnF, phnG, phnJ, phnK, phnL,  
20 phnM, phnN, phnO, phnP, eda, rpiB, zwf, mopA, pstA, pstB, pstC, pstS, ugpB, ugpA, ugpE, ugpC, ugpQ, dnaK, dnaJ, clpB, rpoE, htpG, ompF, ompC, aspA, gltA, sdhB, aceB and aceK, for one or more enzymes of the known threonine biosynthesis pathway or enzymes of anaplerotic metabolism or enzymes for  
25 the production of reduced nicotinamide adenine dinucleotide phosphate or enzymes of glycolysis or PTS enzymes or enzymes of sulfur metabolism to be enhanced.

Thus, for example, at the same time one or more of the genes chosen from the group consisting of

30 • the thrABC operon which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase (US-A-4,278,765),

- the pyc gene of *Corynebacterium glutamicum* which codes for pyruvate carboxylase (WO 99/18228),
- the pps gene which codes for phosphoenol pyruvate synthase (Molecular and General Genetics 231(2): 332-336  
5 (1992)),
- the ppc gene which codes for phosphoenol pyruvate carboxylase (Gene 31: 279-283 (1984)),
- the pntA and pntB genes which code for transhydrogenase (European Journal of Biochemistry 158: 647-653 (1986)),
- 10 • the rhtB gene which imparts homoserine resistance (EP-A-0 994 190),
- the mqo gene which codes for malate:quinone oxidoreductase (WO 02/06459),
- the rhtC gene which imparts threonine resistance (EP-A-1  
15 013 765),
- the thrE gene of *Corynebacterium glutamicum* which codes for the threonine export protein (WO 01/92545),
- the gdhA gene which codes for glutamate dehydrogenase (Nucleic Acids Research 11: 5257-5266 (1983); Gene 23:  
20 199-209 (1983)),
- the hns gene which codes for the DNA-binding protein HLP-II (Molecular and General Genetics 212: 199-202 (1988)),
- the pgm gene which codes for phosphoglucomutase (Journal  
25 of Bacteriology 176: 5847-5851 (1994)),
- the fba gene which codes for fructose biphosphate aldolase (Biochemical Journal 257: 529-534 (1989)),
- the ptsH gene of the ptsHIcrr operon which codes for the phosphohistidine protein hexose phosphotransferase of

the phosphotransferase system PTS (Journal of Biological Chemistry 262: 16241-16253 (1987)),

- the ptsI gene of the ptsHIcrr operon which codes for enzyme I of the phosphotransferase system PTS (Journal of Biological Chemistry 262: 16241-16253 (1987)),  
5
- the crr gene of the ptsHIcrr operon which codes for the glucose-specific IIA component of the phosphotransferase system PTS (Journal of Biological Chemistry 262: 16241-16253 (1987)),
- 10 • the ptsG gene which codes for the glucose-specific IIBC component (Journal of Biological Chemistry 261: 16398-16403 (1986)),
- the lrp gene which codes for the regulator of the leucine regulon (Journal of Biological Chemistry 266: 15  
10768-10774 (1991)),
- the mopB gene which codes for 10 Kd chaperone (Journal of Biological Chemistry 261: 12414-12419 (1986)) and is also known by the name groES,  
15
- the ahpC gene of the ahpCF operon which codes for the small sub-unit of alkyl hydroperoxide reductase (Proceedings of the National Academy of Sciences of the United States of America 92: 7617-7621 (1995)),  
20
- the ahpF gene of the ahpCF operon which codes for the large sub-unit of alkyl hydroperoxide reductase (Proceedings of the National Academy of Sciences of the United States of America 92: 7617-7621 (1995)),  
25
- the cysK gene which codes for cysteine synthase A (Journal of Bacteriology 170: 3150-3157 (1988)),
- the cysB gene which codes for the regulator of the cys regulon (Journal of Biological Chemistry 262: 5999-6005 (1987)),  
30

- the cysJ gene of the cysJIH operon which codes for the flavoprotein of NADPH sulfite reductase (Journal of Biological Chemistry 264: 15796-15808 (1989), Journal of Biological Chemistry 264: 15726-15737 (1989)),
- 5 • the cysI gene of the cysJIH operon which codes for the haemoprotein of NADPH sulfite reductase (Journal of Biological Chemistry 264: 15796-15808 (1989), Journal of Biological Chemistry 264: 15726-15737 (1989)),
- the cysH gene of the cysJIH operon which codes for adenylyl sulfate reductase (Journal of Biological Chemistry 264: 15796-15808 (1989), Journal of Biological Chemistry 264: 15726-15737 (1989)),
- 10 • the malE gene which codes for the periplasmic binding protein of maltose transport (Journal of Biological Chemistry 259 (16): 10606-10613 (1984)),
- the phoB gene of the phoBR operon which codes for the positive regulator PhoB of the pho regulon (Journal of Molecular Biology 190 (1): 37-44 (1986)),
- 15 • the phoR gene of the phoBR operon which codes for the sensor protein of the pho regulon (Journal of Molecular Biology 192 (3): 549-556 (1986)),
- the phoE gene which codes for protein E of the outer cell membrane (Journal of Molecular Biology 163 (4): 513-532 (1983)),
- 20 • the pykF gene which codes for fructose-stimulated pyruvate kinase I (Journal of Bacteriology 177 (19): 5719-5722 (1995)),
- the pfkB gene which codes for 6-phosphofructokinase II (Gene 28 (3): 337-342 (1984)),
- 25 • the talB gene which codes for transaldolase B (Journal of Bacteriology 177 (20): 5930-5936 (1995)),

- the rseA gene of the rseABC operon which codes for a membrane protein with anti-sigmaE activity (Molecular Microbiology 24 (2): 355-371 (1997)),
- the rseC gene of the rseABC operon which codes for a global regulator of the sigmaE factor (Molecular Microbiology 24 (2): 355-371 (1997)),
- the sodA gene which codes for superoxide dismutase (Journal of Bacteriology 155 (3): 1078-1087 (1983)),
- the sucA gene of the sucABCD operon which codes for the decarboxylase sub-unit of 2-ketoglutarate dehydrogenase (European Journal of Biochemistry 141 (2): 351-359 (1984)),
- the sucB gene of the sucABCD operon which codes for the dihydrolipoyltranssuccinase E2 sub-unit of 2-ketoglutarate dehydrogenase (European Journal of Biochemistry 141 (2): 361-374 (1984)),
- the sucC gene of the sucABCD operon which codes for the  $\beta$ -sub-unit of succinyl-CoA synthetase (Biochemistry 24 (22): 6245-6252 (1985)) and
- the sucD gene of the sucABCD operon which codes for the  $\alpha$ -sub-unit of succinyl-CoA synthetase (Biochemistry 24 (22): 6245-6252 (1985)),

can be enhanced, in particular over-expressed.

It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, in addition to enhancement of one or more of the genes chosen from the group consisting of phoA, phnC, phnD, phnE, phnF, phnG, phnJ, phnK, phnL, phnM, phnN, phnO, phnP, eda, rpiB, zwf, mopA, pstA, pstB, pstC, pstS, ugpB, ugpA, ugpE, ugpC, ugpQ, dnaK, dnaJ, clpB, rpoE, htpG, ompF, ompC, aspA, gltA, sdhB,

aceB and aceK, for one or more of the genes chosen from the group consisting of

- the tdh gene which codes for threonine dehydrogenase (Journal of Bacteriology 169: 4716-4721 (1987)),
- 5 • the mdh gene which codes for malate dehydrogenase (E.C. 1.1.1.37) (Archives in Microbiology 149: 36-42 (1987)),
- the gene product of the open reading frame (orf) yjfa (Accession Number AAC77180 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA)),
- 10 • the gene product of the open reading frame (orf) ytfP (Accession Number AAC77179 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA)),
- the pckA gene which codes for the enzyme phosphoenol pyruvate carboxykinase (Journal of Bacteriology 172: 7151-7156 (1990)),
- 15 • the poxB gene which codes for pyruvate oxidase (Nucleic Acids Research 14(13): 5449-5460 (1986)),
- the aceA gene which codes for the enzyme isocitrate lyase (Journal of Bacteriology 170: 4528-4536 (1988)),
- 20 • the dgsA gene which codes for the DgsA regulator of the phosphotransferase system (Bioscience, Biotechnology and Biochemistry 59: 256-251 (1995)) and is also known under the name of the mlc gene,
- the fruR gene which codes for the fructose repressor (Molecular and General Genetics 226: 332-336 (1991)) and is also known under the name of the cra gene and
- 25 • the rpoS gene which codes for the sigma<sup>38</sup> factor (WO 01/05939) and is also known under the name of the katF gene,

to be attenuated, in particular eliminated or for the expression thereof to be reduced.

The term "attenuation" in this connection describes the reduction or elimination of the intracellular activity of  
5 one or more enzymes (proteins) in a microorganism which are coded by the corresponding DNA, for example by using a weak promoter or a gene or allele which codes for a corresponding enzyme with a low activity or inactivates the corresponding enzyme (protein) or gene, and optionally  
10 combining these measures.

By attenuation measures, the activity or concentration of the corresponding protein is in general reduced to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein or of  
15 the activity or concentration of the protein in the starting microorganism.

It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, in addition to enhancement of one or more of the genes chosen from the  
20 group consisting of phoA, phnC, phnD, phnE, phnF, phnG, phnJ, phnK, phnL, phnM, phnN, phnO, phnP, eda, rpiB, zwf, mopA, pstA, pstB, pstC, pstS, ugpB, ugpA, ugpE, ugpC, ugpQ, dnaK, dnaJ, clpB, rpoE, htpG, ompF, ompC, aspA, gltA, sdhB, aceB and aceK, to eliminate undesirable side reactions  
25 (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sickyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms produced according to the invention can  
30 be cultured in the batch process (batch culture), the fed batch (feed process) or the repeated fed batch process (repetitive feed process). A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die

Bioverfahrenstechnik [Bioprocess Technology 1. Introduction to Bioprocess Technology (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen [Bioreactors and Peripheral

- 5 Equipment] (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General

- 10 Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and optionally cellulose, oils and fats, such as e.g. soya oil, sunflower

- 15 oil, groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid, can be used as the source of carbon. These substances can be used individually or as

20 a mixture.

Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium

- 25 phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-

- 30 containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as e.g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as sulfur dioxide and vitamins, can be

employed in addition to the abovementioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in 5 during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture.

- 10 Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, e.g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or
- 15 oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 25°C to 45°C, and preferably 30°C to 40°C. Culturing is continued until a maximum of L-amino acids or L-threonine has formed. This target is usually 20 reached within 10 hours to 160 hours.

The analysis of L-amino acids can be carried out by anion exchange chromatography with subsequent ninhydrin derivatization, as described by Spackman et al. (Analytical Chemistry 30: 1190-1206 (1958)), or it can take place by 25 reversed phase HPLC as described by Lindroth et al. (Analytical Chemistry 51: 1167-1174 (1979)).

The process according to the invention is used for the fermentative preparation of L-amino acids, such as, for example, L-threonine, L-isoleucine, L-valine, L-methionine, 30 L-homoserine and L-lysine, in particular L-threonine.

**What is claimed is:**

1. A process for the preparation of L-amino acids, in particular L-threonine, which comprises carrying out the following steps:
  - 5        a) fermentation of microorganisms of the Enterobacteriaceae family which produce the desired L-amino acid and in which one or more of the genes chosen from the group consisting of phoA, phnC, phnD, phnE, phnF, phnG, phnJ, phnK, phnL, phnM, phnN, phnO, phnP, eda, rpiB, zwf, mopA, pstA, pstB, pstC, pstS, ugpB, ugpA, ugpE, ugpC, ugpQ, dnaK, dnaJ, clpB, rpoE, htpG, ompF, ompC, aspA, gltA, sdhB, aceB and aceK, or nucleotide sequences which code for these, is or are enhanced, in particular over-expressed,
  - 10        b) concentration of the desired L-amino acid in the medium or in the cells of the microorganisms, and
  - 15        c) isolation of the desired L-amino acid, constituents of the fermentation broth and/or the biomass in its entirety or portions (> 0 to 100 %) thereof optionally remaining in the product.
- 20        2. A process as claimed in claim 1, wherein microorganisms in which further genes of the biosynthesis pathway of the desired L-amino acid are additionally enhanced are employed.
- 25        3. A process as claimed in claim 1, wherein microorganisms in which the metabolic pathways which reduce the formation of the desired L-amino acid are at least partly eliminated are employed.
- 30        4. A process as claimed in claim 1, wherein the expression of the polynucleotide (s) which code(s) for one or more of the genes chosen from the group consisting of phoA,

phnC, phnD, phnE, phnF, phnG, phnJ, phnK, phnL, phnM, phnN, phnO, phnP, eda, rpiB, zwf, mopA, pstA, pstB, pstC, pstS, ugpB, ugpA, ugpE, ugpC, ugpQ, dnaK, dnaJ, clpB, rpoE, htpG, ompF, ompC, aspA, gltA, sdhB, aceB and aceK is increased.

5. A process as claimed in claim 1, wherein the regulatory and/or catalytic properties of the polypeptides (proteins) for which the polynucleotides phoA, phnC, phnD, phnE, phnF, phnG, phnJ, phnK, phnL, phnM, phnN, phnO, phnP, eda, rpiB, zwf, mopA, pstA, pstB, pstC, pstS, ugpB, ugpA, ugpE, ugpC, ugpQ, dnaK, dnaJ, clpB, rpoE, htpG, ompF, ompC, aspA, gltA, sdhB, aceB and aceK code are improved or increased.
10. A process as claimed in claim 1, wherein the regulatory and/or catalytic properties of the polypeptides (proteins) for which the polynucleotides phoA, phnC, phnD, phnE, phnF, phnG, phnJ, phnK, phnL, phnM, phnN, phnO, phnP, eda, rpiB, zwf, mopA, pstA, pstB, pstC, pstS, ugpB, ugpA, ugpE, ugpC, ugpQ, dnaK, dnaJ, clpB, rpoE, htpG, ompF, ompC, aspA, gltA, sdhB, aceB and aceK code are improved or increased.
15. A process as claimed in claim 1, wherein, for the preparation of L-amino acids, microorganisms of the Enterobacteriaceae family in which in addition at the same time one or more of the genes chosen from the group consisting of:
20. 6.1 the thrABC operon which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase,
- 6.2 the pyc gene which codes for pyruvate carboxylase,
25. 6.3 the pps gene which codes for phosphoenol pyruvate synthase,
- 6.4 the ppc gene which codes for phosphoenol pyruvate carboxylase,
- 6.5 the pntA and pntB genes which code for transhydrogenase,
30. 6.6 the rhtB gene which imparts homoserine resistance,

- 6.7 the *mgo* gene which codes for malate:quinone oxidoreductase,
- 6.8 the *rhtC* gene which imparts threonine resistance,
- 5 6.9 the *thrE* gene which codes for the threonine export protein,
- 6.10 the *gdhA* gene which codes for glutamate dehydrogenase,
- 10 6.11 the *hns* gene which codes for the DNA-binding protein HLP-II,
- 6.12 the *pgm* gene which codes for phosphoglucomutase,
- 6.13 the *fba* gene which codes for fructose biphosphate aldolase,
- 15 6.14 the *ptsH* gene which codes for the phosphohistidine protein hexose phosphotransferase,
- 6.15 the *ptsI* gene which codes for enzyme I of the phosphotransferase system,
- 20 6.16 the *crr* gene which codes for the glucose-specific IIA component,
- 6.17 the *ptsG* gene which codes for the glucose-specific IIBC component,
- 25 6.18 the *lrp* gene which codes for the regulator of the leucine regulon,
- 6.19 the *mopB* gene which codes for 10 Kd chaperone,
- 6.20 the *ahpC* gene which codes for the small sub-unit of alkyl hydroperoxide reductase,

- 6.21 the ahpF gene which codes for the large sub-unit of alkyl hydroperoxide reductase,
- 6.22 the cysK gene which codes for cysteine synthase A,
- 5 6.23 the cysB gene which codes for the regulator of the cys regulon,
- 6.24 the cysJ gene which codes for the flavoprotein of NADPH sulfite reductase,
- 10 6.25 the cysI gene which codes for the haemoprotein of NADPH sulfite reductase,
- 6.26 the cysH gene which codes for adenylyl sulfate reductase,
- 6.27 the malE gene which codes for the periplasmic binding protein of maltose transport,
- 15 6.28 the phoB gene which codes for the positive regulator PhoB of the pho regulon,
- 6.29 the phoR gene which codes for the sensor protein of the pho regulon,
- 20 6.30 the phoE gene which codes for protein E of outer cell membrane,
- 6.31 the pykF gene which codes for fructose-stimulated pyruvate kinase I,
- 6.32 the pfkB gene which codes for 6-phosphofructokinase II,
- 25 6.33 the talB gene which codes for transaldolase B,
- 6.34 the rseA gene which codes for a membrane protein which acts as a negative regulator on sigmaE activity,

- 6.35 the rseC gene which codes for a global regulator of the sigmaE factor,
  - 6.36 the sodA gene which codes for superoxide dismutase,
  - 5 6.37 the sucA gene which codes for the decarboxylase sub-unit of 2-ketoglutarate dehydrogenase,
  - 6.38 the sucB gene which codes for the dihydrolipoyltranssuccinase E2 sub-unit of 2-ketoglutarate dehydrogenase,
  - 10 6.39 the sucC gene which codes for the  $\beta$ -sub-unit of succinyl-CoA synthetase,
  - 6.40 the sucD gene which codes for the  $\alpha$ -sub-unit of succinyl-CoA synthetase,
- is or are enhanced, in particular over-expressed, are  
15 fermented.
7. A process as claimed in claim 1, wherein, for the preparation of L-amino acids, microorganisms of the Enterobacteriaceae family in which in addition at the same time one or more of the genes chosen from the  
20 group consisting of:
- 7.1 the tdh gene which codes for threonine dehydrogenase,
  - 7.2 the mdh gene which codes for malate dehydrogenase,
  - 25 7.3 the gene product of the open reading frame (orf) yjfa,
  - 7.4 the gene product of the open reading frame (orf) ytfp,

- 7.5 the pckA gene which codes for phosphoenol pyruvate carboxykinase,
- 7.6 the poxB gene which codes for pyruvate oxidase,
- 7.7 the aceA gene which codes for isocitrate lyase,
- 5 7.8 the dgsA gene which codes for the DgsA regulator of the phosphotransferase system,
- 7.9 the fruR gene which codes for the fructose repressor,
- 10 7.10 the rpoS gene which codes for the sigma<sup>38</sup> factor
- is or are attenuated, in particular eliminated or reduced in expression, are fermented.

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**WO 03/008607 A3**

(54) Title: PROCESS FOR THE PREPARATION OF L-AMINO ACIDS USING STRAINS OF THE ENTEROBACTERIACEAE FAMILY

(57) Abstract: The invention relates to a process for the preparation of L-amino acids, in particular L-threonine, in which the following steps are carried out: a) fermentation of microorganisms of the Enterobacteriaceae family which produce the desired L-amino acid and in which at least one or more of the genes chosen from the group consisting of phoA, phnC, phnD, phnE, phnF, phnG, phnJ, phnK, phnL, phnM, phnN, phnO, phnP, eda, rpiB, zwf, mopA, pstA, pstB, pstC, pstS, ugpB, ugpA, ugpE, ugpC, ugpQ, dnaK, dnaJ, clpB, rpoE, htpG, ompF, ompC, aspA, gltA, sdhB, aceB and aceK, or nucleotide sequences which code for these, is or are enhanced, in particular over-expressed, b) concentration of the desired L-amino acid in the medium or in the cells of the bacteria, and c) isolation of the desired L-amino acid.

## INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 02/07356
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## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/31	C12N15/52	C12P13/08	C12P13/04	C07K14/245
//(C12P13/08,C12R1:19)				

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12P C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, EMBASE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>WANNER B L: "Gene regulation by phosphate in enteric bacteria." JOURNAL OF CELLULAR BIOCHEMISTRY, vol. 51, no. 1, January 1993 (1993-01), pages 47-54, XP008018017 ISSN: 0730-2312 the whole document</p> <p>-----</p> <p>TORRIANI A: "From cell membrane to nucleotides: the phosphate regulon in Escherichia coli." BIOESSAYS: NEWS AND REVIEWS IN MOLECULAR, CELLULAR AND DEVELOPMENTAL BIOLOGY, vol. 12, no. 8, August 1990 (1990-08), pages 371-376, XP008018018 ISSN: 0265-9247 the whole document</p> <p>-----</p> <p>-/-</p>	1-7
A		1-7

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

## \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
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## INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 994 190 A (AJINOMOTO KK) 19 April 2000 (2000-04-19) the whole document example 4 claims 6,7,10,11 -----	1-7
A	WO 99 53035 A (ALTMAN ELLIOT ;GOKARN RAVI R (US); EITEMAN MARK A (US); UNIV GEORG) 21 October 1999 (1999-10-21) page 5, lines 20-24 examples 4,7,9,10 claims 31,38,41,49 figures 1,4 -----	1-7
A	MICHAL G: "Biochemical pathways: an atlas of biochemistry and molecular biology" 1999, . JOHN WILEY & SONS INC. AND SPEKTRUM AKADEMISCHER VERLAG , NEW YORK - HEIDELBERG XP002242199 ISBN: 0-471-33130-9 figure 3.8-2  figures 4.2-1, 4.5-1 and 4.5-2 -----	1-7
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### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
  
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-7 all partially

#### Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
- No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

Invention 1, claims 1-7 (all partially)

A process for the preparation of L-amino acids, in particular L-threonine, comprising the steps of a) fermenting a microorganism of the Enterobacteriaceae family which produces the desired L-amino acid and in which the phoA gene is enhanced, in particular overexpressed, b) concentrating and (c) isolating the desired L-amino acid, as well as a process as said in which additional genes are enhanced and/or attenuated.

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Inventions 2-38, claims 1-7 (all partially)

As invention 1, but with the following genes being enhanced, respectively: phnC, phnD, phnE, phnF, phnG, phnJ, phnK, phnL, phnM, phnN, phnO, phnP, eda, rpiB, zwf, mopA, pstA, pstB, pstC, pstS, ugpB, ugpA, ugpE, ugpC, ugpQ, dnaK, dnaJ, clpB, rpoE, htpG, ompF, ompC, aspA, gltA, sdhB, aceB and aceK.

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